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Interaction of *Francisella asiatica* with Tilapia (*Oreochromis niloticus*) Innate Immunity[▽]

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Members of the genus *Francisella* are facultative intracellular bacteria that cause important diseases in a wide variety of animals worldwide, including humans and fish. Several genes that are important for intramacrophage survival have been identified, including the *iglC* gene, which is found in the *iglABCD* operon in the *Francisella* sp. pathogenicity island (FPI). In the present study, we examined the interaction of wild-type *Francisella asiatica* and a Δ*iglC* mutant strain with fish serum and head kidney-derived macrophages (HKDM). Both the wild-type and the mutant strains were resistant to killing by normal and heat-inactivated sera. The wild-type *F. asiatica* is able to invade tilapia head kidney-derived macrophages and replicate vigorously within them, causing apoptosis and cytotoxicity in the macrophages at 24 and 36 h postinfection. The Δ*iglC* mutant, however, is defective for survival, replication, and the ability to cause cytotoxicity in HKDM, but the ability is restored when the mutant is complemented with the *iglC* gene. Uptake by the HKDM was mediated partially by complement and partially by macrophage mannose receptors, as demonstrated by *in vitro* assays. Light and electron microscopy analysis of the infected macrophages revealed intracellular bacteria present in a tight vacuole at 2 h postinoculation and the presence of numerous bacteria in spacious vacuoles at 12 h postinfection, with some bacteria free in the cytoplasm.

Francsiella asiatica and *Francsiella noatunensis* are recently described members of the genus *Francisella* (42, 43). *Francisella noatunensis* isolates were recovered from diseased cultured cod (*Gadus morhua*) in Norway (42, 43). *Francsiella asiatica* (isolate Ehime-1) was recovered from diseased three line grunt (*Parapristipoma trilineatum*) in Japan and was the isolate used to describe the new species (31, 43). In the last 5 years the bacterium has caused substantial mortality in tilapia (*Oreochromis* spp.) and other important warm and cold water species cultured in the United States (including Hawaii); Taiwan; Costa Rica, Chile, and other parts of Latin America; Norway; and Japan (14, 30, 31, 40, 42, 48, 49, 57). In Taiwan, reports of rickettsia-like organisms causing disease in fresh, brackish, and salt water pond-cultured tilapia can be tracked to the early 1990s, and in recent years, several farms have reported mortalities of up to 95% due to this pathogen (30). In Hawaii and in Costa Rica and other parts of Latin America, a similar situation has been present since 2004, when mortalities of up to 90% in brackish water- and freshwater-cultured tilapia were reported (40, 57). Moreover, the bacterium not only has been isolated and found to cause disease in important worldwide culture species such as tilapia, three line grunt, cod, and Atlantic salmon (*Salmo salar*) but has been found in wild fish such as the guapote (*Cichlasoma managuense*) in Costa Rica and other parts of Latin America and wild mackerel (*Scomber scombrus*) and cod in Norway (14, 30, 31, 40, 42, 48, 49, 57).

Fish francisellosis is an emergent disease of a wide variety of fish species. The disease can present as an acute syndrome with few clinical signs and high mortality or as a subacute to chronic syndrome with nonspecific clinical signs, including anorexia, exophthalmia, and anemia. Upon macroscopic and microscopic examination, internal organs are enlarged and contain widespread multifocal white nodules. Histological examination reveals the presence of multifocal granulomatous lesions containing numerous small, pleomorphic, coccobacilli (57). In the majority of the cases, PCR and sequence comparison of the 16S rRNA place the organism at 97% similarity to *Francsiella tularensis* and 98% similarity to *Francsiella philomiragia* (14, 30, 31, 40, 42, 48, 49, 50, 57).

Francisella tularensis is the most important species belonging to this genus (1, 21, 56). Besides being an important animal pathogen, *F. tularensis* is a zoonotic agent that has received considerable study as a potential bioterrorism agent because it has a high infectivity rate and multiple infectious routes (33, 46). The genetic basis of *F. tularensis* virulence is still poorly understood, although several virulence determinants have been identified (7, 30, 45). Previous studies described the intracellular localization, survival, and replication of *F. tularensis* in polymorphonuclear leukocytes (PMNs), macrophages, adherent mouse peritoneal cells, the mouse macrophage-like cell line J774A.1, and the human macrophage cell line THP-1 and include the ultimate escape from the phagolysosome into the cytoplasm (1, 3, 4, 10, 20, 26, 52). Some of the most interesting genes involved in this process are the genes of the intracellular growth locus, *iglA*, *iglB*, *iglC*, and *iglD*, which are present as part of a 30-kb pathogenicity island (7, 46). The functions of the conserved proteins corresponding to these genes are elusive, although the Igl proteins appear to be essential for the ability of *F. tularensis* to survive inside macrophages and cause disease (15, 17, 19, 20, 25, 35, 37, 45, 53). Recent data showed that IglA and IglB are part of a novel *Francisella* pathogenicity island

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(FPI)-encoded type 6 secretion system (T6SS) (39, 46). Mutations of the *iglABCD* genes in *F. tularensis* resulted in decreased pathogenicity both *in vivo* and *in vitro* in mammalian and insect tissues and cell lines (20, 36, 45, 61).

Homologues to the *F. tularensis iglA*, *iglB*, *iglC*, and *iglD* genes are present in *F. asiatica* strain LADL 07-285A, which was isolated from diseased tilapia. DNA sequence comparison between the *F. asiatica* LADL 07-285A, *F. philomiragia* subsp. *philomiragia*, and *F. tularensis* subsp. *novicida* U112 *iglABCD* operons revealed 94% identity to *F. philomiragia* and 83% identity to *F. tularensis* subsp. *novicida*. It was previously demonstrated that as few as 23 *F. asiatica* bacteria injected in the peritoneum are capable of causing mortalities in tilapia nilotica (*Oreochromis niloticus*) and that even fewer are enough to cause serious pathological lesions in important organs such as the head kidney and spleen (58), but the pathogenic mechanisms that underlie its remarkable infectivity and its capacity to cause disease in a broad range of fish hosts are poorly known. In previous work, however, an insertion mutation in the *iglC* gene of *F. asiatica* LADL 07-285A was constructed by allelic exchange, and the Δ*iglC* mutant was found to be attenuated following intraperitoneal and immersion challenges in tilapia (58).

In the present study we use *F. asiatica* LADL 07-285A to investigate the interaction between this emergent pathogen and innate immunity in tilapia. We demonstrate that the *F. asiatica* wild-type isolate is resistant to serum killing; is able to enter, survive in, and replicate in tilapia head kidney-derived macrophages (HKDM); and ultimately kills the cell by inducing apoptosis. Mutation of the *iglC* gene, however, makes *F. asiatica* defective for intramacrophagic survival and replication, as well as for induction of apoptotic caspase 3 and 7 cleavage and cytotoxicity, but does not affect its ability to survive in serum. Finally, we demonstrate that complementation of the IglC protein restores virulence, the proapoptotic features of the defective mutant, and cytotoxicity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Francisella asiatica* LADL 07-285A was isolated from cultured tilapia (*Oreochromis* sp.) and was described in previous work (57). The Δ*iglC* mutant isolate was made by homologous recombination using a PCR product, and its attenuation was demonstrated *in vivo* (58). *Francisella asiatica* was grown on cystine heart agar supplemented with bovine hemoglobin solution (CHAH) (Becton Dickinson [BD] BBL, Sparks, MD) for 48 h at 28°C or in Mueller-Hinton II cation-adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD) and 0.1% glucose (MMH) (5). Broth cultures were grown overnight at 25°C in a shaker at 175 rpm, and bacteria were frozen at −80°C in the broth medium containing 20% glycerol for later use.

The *Escherichia coli* QC 779 serum-sensitive isolate and *E. coli* strain DH5α were grown using Luria-Bertani broth or agar for 16 to 24 h at 37°C. When needed, kanamycin and/or tetracycline was added to the agar and broth media at concentrations of 15 μg/ml and 10 μg/ml, respectively.

Growth curves of the *F. asiatica* wild-type and Δ*iglC* mutant strains were determined by inoculating duplicate culture tubes containing 50 ml MMH broth with 500 μl of overnight broth cultures adjusted to an optical density at 600 nm (OD_{600}) of 0.8 for each strain. The cultures were incubated at 25°C for 24 h on an orbital shaker (200 rpm), and growth was monitored every 2 h by measuring the optical density at 600 nm.

Construction of complementing IglC plasmid. Briefly, for complementation, plasmid pKEK894 (63) was used to clone the *iglC* gene. The *iglC* gene was amplified by PCR from *F. asiatica* LADL 07-285A genomic DNA with primers FAcoI-*iglC*-Comp-F (5′AACGCGCCATGGGTATGAATGAAATGATAACA AGAC-3′) and FEcoRI-*iglC*-Comp-R (5′GCGCGGAATTCCGATCTTACTA TGCAGAT-3′). The PCR fragment was digested with NcoI and EcoRI, and ligated into NcoI- and EcoRI-digested pKEK894, to form pKEK-Fa*iglC*. The pKEK-Fa*iglC* plasmid was then electroporated into *E. coli* ΔH5a, reisolated, and electroporated into *F. asiatica* wild-type LADL 07-285A and the Δ*iglC* mutant as previously described (58).

Fish. Adult tilapia (*Oreochromis niloticus*) (mean weight, 342 g) were obtained from an inland farm with no history of fish francisellosis. Fish were acclimated for a minimum of 2 months in a recirculating system at 25°C under optimum water quality conditions. Ten fish were euthanized using 100 mg/ml of tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) and analyzed for evidence of *Francisella* by clinical examination, bacteriological isolation, and PCR (57).

Bactericidal activity of normal and heat-inactivated sera. Blood was collected from 10 adult tilapia by caudal venipuncture using 3 ml red-top Vacutainer tubes (BD Vacutainer Systems, Franklin Lakes, NJ). Prior to bleeding, the fish were anesthetized with 100 mg/ml MS-222. Blood was allowed to clot for 4 h at 4°C before serum was collected by centrifugation at 3,000 × *g* for 10 min. A subsample of the collected serum was heated in a water bath at 55°C for 30 min to inactivate complement.

Wild-type *Francisella asiatica*, the Δ*iglC* mutant, and *E. coli* isolate QC 779 (serum sensitive) were cultured as described above. Bacteria were adjusted to a concentration of 1×10^7 CFU/ml in PBS, and equal volumes of the bacterial isolates and either normal or heat-inactivated tilapia serum were combined and incubated at room temperature. At 0, 1, and 2 h, subsamples were collected, serially diluted in PBS, and spotted onto either CHAH (*F. asiatica*) or LB (*E. coli*) plates for determination of CFU numbers.

Macrophage media. An optimal medium for culture of tilapia macrophages was designed based on previously published media for cultivation of channel catfish and hybrid striped bass macrophages (16, 22) but with an osmolality of 320 mosmol/kg H_2O to match tilapia serum osmolality. The complete tilapia macrophage medium (CTMM) consisted of Roswell Park Memorial Institute (RPMI) medium 1640 (GIBCO, Invitrogen Corp., Carlsbad, CA) with 14 mM HEPES buffer (GIBCO, Invitrogen Corp.), 0.3% sodium bicarbonate (GIBCO, Invitrogen Corp.), 0.05 mM 2-beta-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 5% heat-inactivated, pooled tilapia serum.

Growth of *F. asiatica* in macrophage culture media. To evaluate the reliability of using various media for an *in vitro* intramacrophagic survival assay, growth of *F. asiatica* LADL 07-285A was compared in Dulbecco modified Eagle medium containing 10% tilapia serum (DMEM), CTMM, CTMM with the addition of 10 μg/ml of gentamicin, and MMH. Triplicate wells of a 96-well microtiter plate were inoculated with 200 μl each of medium, containing approximately 2.3×10^6 CFU/ml. Bacteria growth was measured over a period of 24 h by plating serial dilutions on CHAH.

Collection and cultivation of head kidney-derived macrophages. Previous protocols were modified for the collection and culture of tilapia macrophages (16, 44, 55). Briefly, fish were anesthetized with MS-222 and bled from the caudal vein to collect autologous serum. Anterior kidneys were aseptically removed, and the cells were dissociated by passage through a double stainless steel mesh (280 and 140 μm) cell dissociation sieve (Sigma Chemical Co.). Dissociated cells from individual fish were suspended in CTMM. An isosmotic Percoll gradient was prepared following previous published protocols with several modifications (24). The isosmotic Percoll gradient consisted of 9.25 parts of Percoll (Amersham Bioscience, Sweden) and 0.750 parts of 10× phosphate buffer solution (pH 7.1). A Percoll density gradient was prepared in a centrifuge tube by layering a 51% Percoll solution (51% isosmotic Percoll, 49% CTMM) below a 34% Percoll solution (34% isosmotic Percoll, 66% CTMM). The macrophage cell suspension was layered on top of the gradient and was subjected to centrifugation at 400 × *g* for 25 min at 4°C with medium acceleration and low deceleration. The macrophages were collected from the gradient interface and washed twice in CTTM at 400 × *g*, and viability counts were determined using trypan blue dye exclusion. Purification of HKDM was confirmed by nonspecific esterase and Sudan black staining (23), and purity of the samples was >90%. Cells were adjusted to 1×10^7 cells/ml, and 100 μl of the suspension was aliquoted into each well of 96-well microtiter plates coated with poly-D-lysine (BD Biosciences, Bedford, MA). Macrophages were allowed to adhere for 4 h (4h-HKDM) or 5 days (5d-HKDM) at 25°C with 5% CO_2, after which nonadherent cells were removed with three washes of warm CTMM and fresh CTMM was added. In certain experiments, soluble mannan was used to block the mannose receptors (MR) on HKDM as previously described (6). When used, mannan (5 mg/ml) was incubated with HKDM for 30 min at 25°C prior to the addition of bacteria.

Intramacrophage survival assays. To infect tilapia HKDM, a modification of previous protocols was used (17, 45, 53). Briefly, 4-h or 5-day cultures of tilapia head kidney macrophages in 96-well plates containing 1×10^5 to 5×10^5 cells/well were used. *Francisella asiatica* LADL 07-285A was grown for a period

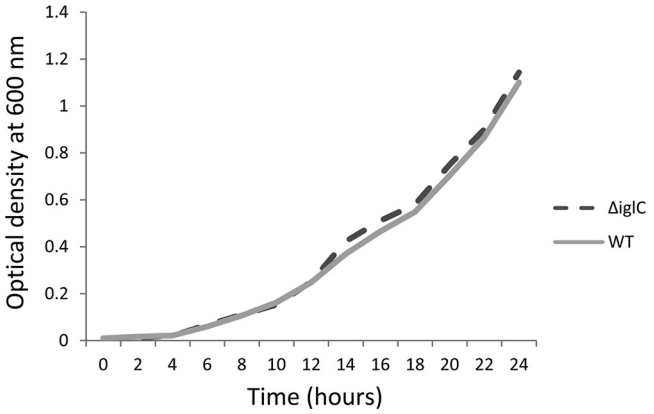

FIG. 1. Growth curves of *Francisella asiatica* wild-type (WT) LADL 07-285A and Δ*igl*C strains. Each strain was grown in MMH broth 25°C, and growth was monitored by determining the optical density at 600 nm.

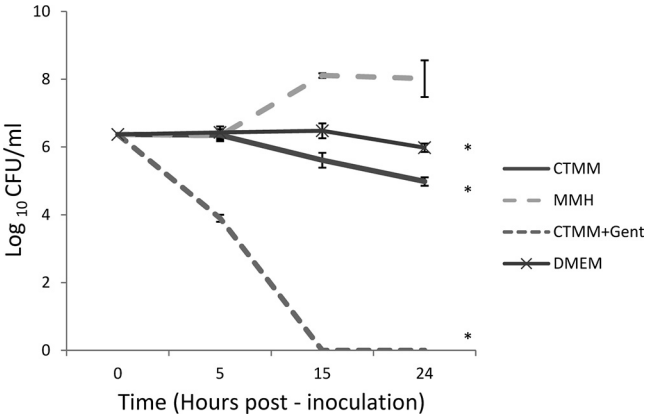

FIG. 2. *In vitro* growth of *Francisella asiatica* LADL 07-285A. LADL 07-285A was cultured in modified Mueller-Hinton broth (MMH), complete tilapia macrophage medium (CTMM), Dulbecco's modified Eagle's medium (DMEM), or CTMM with 10 μg/ml gentamicin (Gent) for 24 h with 5% CO_2. Samples were removed at 5, 15, and 24 h postinoculation and then assayed for bacterial CFU by serial dilution in PBS and plating on CHAH plates. The error bars represent standard errors for triplicate samples, and the results shown are representative of three independent experiments. Significant differences between bacterial growth in CTMM, DMEM, and CTMM with gentamicin and growth in MMH are marked (*, $P < 0.05$).

of 8 h in MMH at 25°C. The OD_{600} of the culture was determined, and the cells were adjusted to an estimated final concentration of 5×10^8 CFU/ml, based on an OD/CFU standard curve. One-milliliter aliquots of the bacterial suspension were pelleted at 10,000 × *g* for 5 min in an Eppendorf 5415 D centrifuge (Eppendorf-Brinkman, Westbury, NY), and the pellet was resuspended in either 1 ml of normal autologous serum (NS), 1 ml of heat-inactivated autologous serum (HINS), or 1 ml of PBS. Tenfold serial dilutions were plated on CHAH after incubation to determine actual CFU/ml. After a 30-min incubation, the 96-well plate was inoculated with 10 μl of opsonized bacteria per well to achieve a multiplicity of infection (MOI) of 50 bacteria to 1 macrophage. The plates were centrifuged for 5 min at 400 × *g* to synchronize bacterial contact with macrophages. Following 2 h of incubation at 25°C with 5% CO_2, the cells were washed three times with warm medium (25°C) and further incubated with fresh medium for 0, 12, 24, or 36 h. Cells in three wells were lysed by the addition of 100 μl of 1% saponin in PBS at each time point. The lysates were serially diluted and spread onto CHAH plates to determine viable counts. Experiments were performed in triplicate on a minimum of three separate occasions to affirm the reliability of the results.

Detection of *F. asiatica*-mediated cytotoxicity. Cytotoxicity was assessed by measuring the release of cytosolic lactate dehydrogenase (LDH) into the supernatant, which reflects a loss of plasma membrane integrity in infected cells. Cytosolic LDH levels were measured using the colorimetric Cytotox 96 kit (Promega, Madison, WI) according to the manufacturer's instructions. The percentage of cytotoxicity was calculated as 100 × [(experimental release − spontaneous release)]/[total release − spontaneous release)], where spontaneous release is the amount of LDH activity in the supernatant of uninfected cells and total release is the activity in cell lysates.

Caspase activity assay. The Apo-ONE homogeneous caspase-3/7 assay (Promega, Madison, WI) was used to measure the activity of caspase-3 and -7 in infected and uninfected HKDM, following the manufacturer's instructions. Members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in eukaryotic cells (34, 35). The Apo-ONE homogeneous caspase-3/7 assay provides a profluorescent substrate with an optimized bifunctional cell lysis/activity buffer for caspase-3/7 (DEVDase) activity assays. The percentage of apoptosis was calculated as 100 × [(experimental release − spontaneous release)]/[total release − spontaneous release)], where spontaneous release is the amount of caspase-3/7 activity in the supernatant of uninfected cells and total release is the activity in cells previously exposed to etoposide (MBL International Corporation, Boburn, MA), following the manufacturer's recommendations to induce 100% apoptosis in the cells.

Electron microscopy. Tilapia head kidney-derived macrophages were attached to 13-mm tissue-culture treated Therminox coverslips (Nalge Nunc, Rochester, NY); infected at an MOI of 50:1; incubated for 2, 6, or 12 h; and processed for transmission electron microscopy. Briefly, primary fixation was for 6 h at room temperature in 1.25% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were postfixed for 1 h in 1% osmium tetraoxide (OsO_4) in distilled water and stained for 2 h with 2% uranyl acetate in 0.2 M sodium acetate buffer, pH 3.5. Ethanol-dehydrated cells were infiltrated and then embedded in epoxy resin. Ultrathin sections were cut on a Sorvall model MT600 ultramicrotome, mounted on 300-mesh copper grids, and stained for 10 min with 5% uranyl acetate in distilled water. Sections were washed three times with double-distilled water and then stained for 2 min with lead citrate. Stained sections were examined on a Zeiss model EM 10C microscope at various magnifications.

Statistical analysis. The experimental design was completely randomized, with a factorial arrangement of treatments. Data were analyzed by the general linear models procedure (PROC GLM) in the Statistical Analysis System after a $\log_{10}$ transformation of the numbers of CFU recovered per well (SAS Institute, Inc., 2003). When the overall model indicated significance ($P < 0.05$), Scheffe's test was used for pairwise comparison of main effects and a least-squares means procedure was used for pairwise comparison of interaction effects.

RESULTS

The *Francisella asiatica* Δ*igl*C mutant grows at the same rate as the wild type in medium. There were no significant differences in the growth curves of the *F. asiatica* 07-285A wild-type and mutant Δ*igl*C strains when grown in MMH broth at 25°C (Fig. 1), indicating that *in vivo* effects were not due to a growth defect in the Δ*iglC* mutant.

CTMM does not support the growth of *F. asiatica*. As demonstrated in Fig. 2, neither DMEM with the addition of 10% heat-inactivated heterologous tilapia serum nor complete tilapia macrophage medium (CTMM) was a favorable environment for *Francisella* growth. *Francisella asiatica* incubated in MMH showed exponential growth after the same incubation period. This finding is in congruence with previous work done with *F. tularensis* isolates, in which the relative inability of *Francisella* to grow extracellularly in macrophage cultures allowed the use of CTMM or DMEM in an *in vitro* assay without the presence of antibiotics in the medium (4, 17, 45, 53).

Both the *F. asiatica* wild-type and Δ*iglC* mutant strains are resistant to serum killing. Both the *F. asiatica* wild-type and mutant Δ*iglC* strains demonstrated complete resistance to serum killing by both heat-inactivated and normal serum, as the

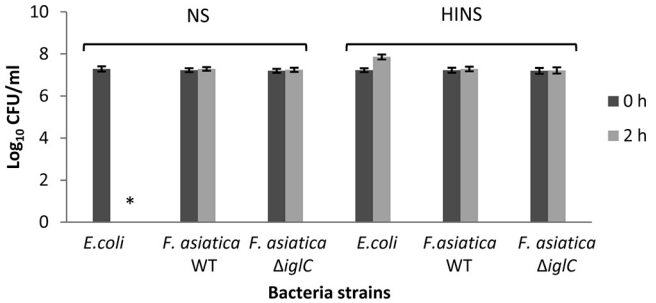

FIG. 3. Survival and growth of *Francisella asiatica* LADL 07-285A wild-type and Δ*iglC* strains and *E. coli* in normal serum (NS) and normal serum that had been heat inactivated at 55°C for 30 m (HINS). The error bars represent standard errors for triplicate samples, and the results shown are representative of three independent experiments. Statistically significant differences between different treatments are marked (∗, $P < 0.001$).

number of bacteria reisolated from the wells was similar to or significantly higher than he number inoculated ($P < 0.001$) (Fig. 3). The serum-sensitive *E. coli* isolate used in the assay was undetectable after only 1 h of incubation with the normal serum. When incubated with heat-inactivated serum, no killing was observed, suggesting that the killing is due to the action of complement.

A heat-sensitive serum component and mannose receptors are necessary for efficient uptake of *F. asiatica* isolates by tilapia HKDM. The uptake of *F. asiatica* by tilapia HKDM was assessed in the presence or absence of normal serum and mannan (a competitive inhibitor of macrophage mannose receptors) in the medium in order to provide insight into the receptors that are involved in the recognition and uptake of *F. asiatica*. In both the 4h-HKDM and 5d-HKDM, uptake of *F. asiatica* was significantly greater when NS was used, indicating that a heat-sensitive component of the serum, most likely complement, was an important mediator of uptake (Fig. 4). Bacteria opsonized with HINS showed 59 and 96.5% decreases in internalization in 4h-HKDM and 5d-HKDM, respectively, compared to bacteria treated with NS. Similar results were obtained when PBS was used to opsonize the bacteria (data not shown). Once inside the macrophages, both NS- and HINS-treated bacteria increased equally in numbers after 12 h of incubation. Although NS increased the uptake of *F. asiatica* with both 4h-HKDM and 5d-HKDM, bacteria were also taken up efficiently in the absence of complement (Fig. 4).

To determine the contribution of the MR in *F. asiatica* recognition, 4h-HKDM and 5d-HKDM were preincubated with soluble mannan, a competitive inhibitor of the MR (6). Mannan pretreatment of HKDM populations significantly decreased internalization of *F. asiatica* in both NS- and HINS-opsonized bacteria. Preincubation of 4h-HKDM with mannan decreased the uptake of *F. asiatica* by 46 and 75% in NS- and HINS-opsonized bacteria, respectively, compared to uptake of bacteria opsonized with NS in non-mannan-treated HKDM (Fig. 4, left panels). On the other hand, preincubation of 5d-HKDM with mannan decreased the uptake of *F. asiatica* by 66 and 99% in NS- and HINS-opsonized bacteria, respectively, compared to uptake of bacteria opsonized with NS in non-mannan-treated HKDM (Fig. 4, right panels). Although not significantly different, uptake of NS-opsonized bacteria by 5d-HKDM was greater than that by 4h-HKDM (Data not shown).

The *Francisella asiatica* wild-type strain survives, replicates, and is cytotoxic in tilapia HKDM, but the Δ*iglC* mutant fails to replicate. To determine whether the *F. asiatica* wild-type strain and the Δ*iglC* mutant were able to survive and replicate in tilapia 4h-HKDM and 5d-HKDM, the numbers of viable bacteria internalized were monitored over a 36-h period in six different experiments. The number of wild-type bacteria recovered from 4h-HKDM after 12, 24, and 36 h increased significantly, by 5-, 45-, and 61-fold, respectively, compared to that at time zero. As shown in Fig. 5, the Δ*iglC* mutant failed to grow ($P < 0.001$), which is consistent with observations of *F. tularensis* Δ*iglC* mutants in mammalian macrophages. Although the macrophages internalized the mutant and wild type equally, the mutant was unable to replicate, but it did persist

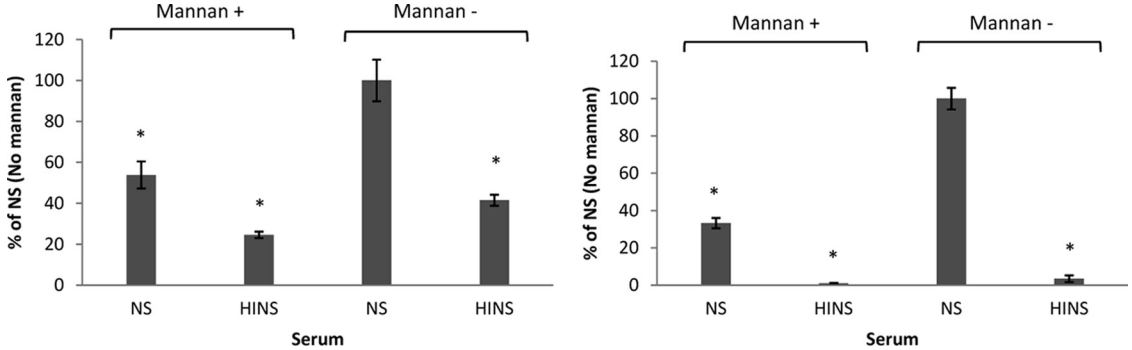

FIG. 4. Phagocytosis of *F. asiatica* LADL 07-285A by tilapia head kidney-derived macrophages (HKDM) is partially mediated by heat-stable serum components and mannose receptors. The 100% level for 4h-HKDM opsonized with NS without mannan pretreatment is 2.65×10^6 CFU/ml. The 100% level for 5d-HKDM opsonized with NS without mannan pretreatment is 4.33×10^6 CFU/ml. At 4 h (left panels) and 5 days (right panels), tilapia HKDM were assessed for their ability to phagocytose *F. asiatica* as described in Materials and Methods. HKDM were incubated with *F. asiatica* (MOI of 1:50 for 2 h) pretreated with either tilapia autologous normal serum (NS) or heat-inactivated normal serum (HINS). HKDM were preincubated with either CTMM containing 5 μg/ml of mannan (Mannan +) or no mannan (Mannan −) for 30 min before addition of bacteria. The error bars represent standard errors for triplicate samples, and the results shown are representative of three independent experiments. Statistically significant differences between bacterial uptake in the different treatments (4h-HKDM and 5d-HKDM opsonized with NS without mannan pretreatment) are marked (∗, $P < 0.001$).

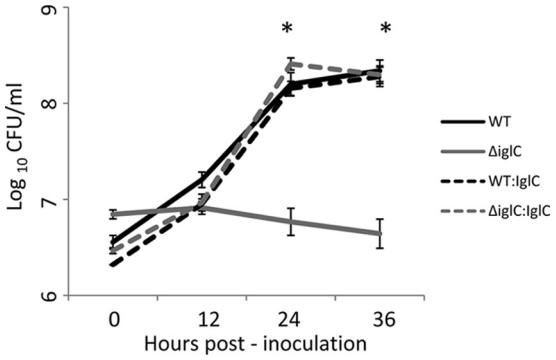

FIG. 5. Growth of *Francisella asiatica* wild-type (WT) LADL 07-285A, the *iglC* mutant (Δ*igl*C), the wild type complemented with IglC (WT:IglC), and the *iglC* mutant complemented with IglC (Δ*igl*C:IglC) in tilapia head kidney-derived macrophages. The error bars represent standard errors for triplicate samples, and the results shown are representative of six independent experiments. Statistically significant differences between different treatments are marked (∗, $P < 0.001$).

for more than 36 h with only a slight decline. Similar results were found in 5d-HKDM (data not shown).

By electron microscopy, it was possible to observe heavily infected cells after 6 h postinoculation for the wild type, but after 12 h a large numbers of macrophages detached from the plate. At 2 hours postinoculation, *F. asiatica* was located inside a membrane-bound tight phagocytic vacuole (Fig. 6A to C). After 12 h, the majority of the bacteria were observed inside spacious vacuoles, although some appeared to have escaped to the cytoplasm (Fig. 6D to F).

Cytotoxicity of the wild type and the Δ*iglC* mutant was examined by monitoring cell morphology and LDH release of the tilapia HKDM. The course of the infection was associated with a progressive cellular degeneration following wild-type challenge. The amount of LDH released by HKDM infected with the wild type was significantly greater than the amount released by HKDM challenged with the mutant. As expected, cytotoxicity was time dependent when HKDM were infected with the wild type, as the amount of LDH released by infected HKDM was significantly greater at 48 h postinoculation than at 0 or 12 h postinoculation (Fig. 7).

***F. asiatica* infection is proapoptotic in tilapia head kidney-derived macrophages.** To determine if infection-induced cytotoxicity is associated with apoptosis, we measured the activity of caspases 3 and 7 in infected tilapia HKDM in both wild-type and mutant *iglC* strains. Active caspases participate in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic structural disassembly of dying cells. At 36 h postinfection, tilapia HKDM infected with the Δ*iglC* mutant had similar levels of caspase 3 and 7 activity and behaved similarly to the uninfected control cells, whereas those infected with the wild type showed a significant increase in caspase 3 and 7 activity, which is a hallmark of apoptosis (Fig. 8).

Complementation of the Δ*iglC* mutant of *F. asiatica* restores the intramacrophage growth ability, cytotoxicity, and proapoptotic features. Intracellular growth, cytotoxicity, and caspase 3/7

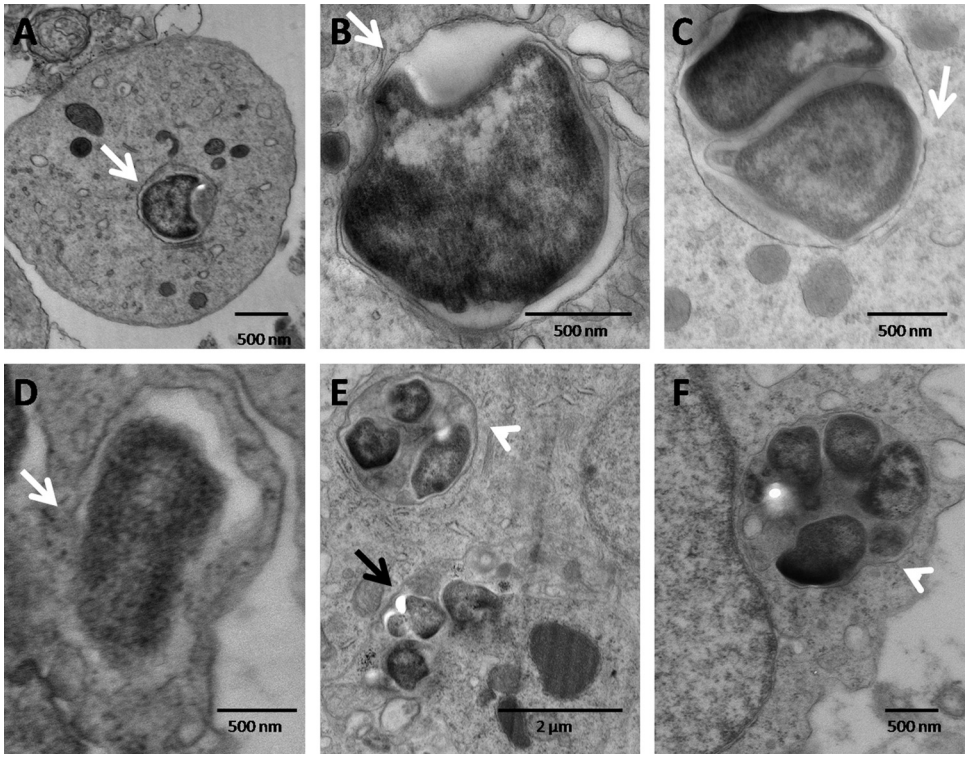

FIG. 6. Transmission electron micrographs of tilapia head kidney-derived macrophages infected with *Francisella asiatica* LADL 07-285A. (A and B). After uptake, the bacteria are located inside a membrane-bounded tight phagocytic vacuole (white arrow) within the macrophage. (C and D) Breakdown of the phagosomal membrane (arrows) appears to allow *F. asiatica* access to the cytoplasm at 6 to 12 h postinfection. (E and F) After 12 h, several bacteria are found inside a spacious vacuole (arrowheads), and some appear to have escaped to the cytoplasm (arrow).

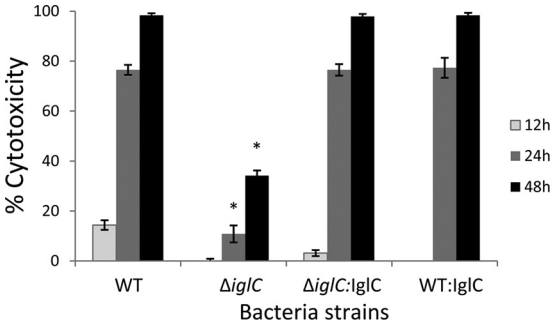

FIG. 7. Cytotoxicity of *Francisella asiatica* wild-type (WT) LADL 07-285A, the *iglC* mutant (Δ*igl*C), the wild type complemented with IglC (WT:IglC), and the *iglC* mutant complemented with IglC (Δ*igl*C:IglC) in tilapia head kidney-derived macrophages at 12, 24, and 48 h postinoculation. Cytotoxicity was assayed by release of LDH from infected cells as described in Materials and Methods. The error bars represent standard errors for triplicate samples, and the results shown are representative of three independent experiments. Statistically significant differences between different treatments are marked (*, $P < 0.001$).

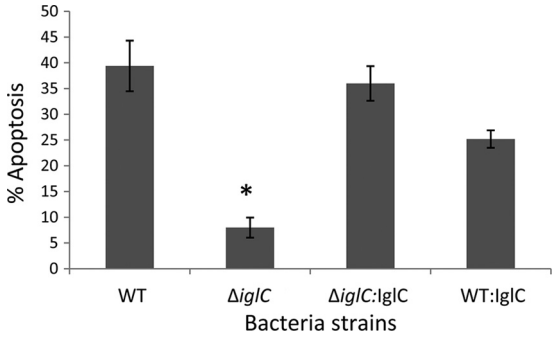

FIG. 8. Tilapia head kidney-derived macrophages display apoptosis at 36 h postinfection with *Francisella asiatica* wild-type (WT) LADL 07-285A, the *iglC* mutant (Δ*igl*C), the wild type complemented with IglC (WT:IglC), and the *iglC* mutant complemented with IglC (Δ*igl*C:IglC). Percent apoptosis was determined by measuring caspase-3/7 activity from infected cells at 36 h postinoculation as described in Materials and Methods. The error bars represent standard errors for triplicate samples, and the results shown are representative of three independent experiments. Statistically significant differences between different treatments are marked (*, $P < 0.001$).

activity were all restored in the Δ*iglC* mutant strain upon complementation (Fig. 5, 7, and 8). The IglC-complemented Δ*iglC* mutant, as well as an IglC-complemented wild-type strain, showed no statistical differences in HKDM intracellular growth, cytotoxicity, or proapoptotic features compared to the wild type.

DISCUSSION

Francisella asiatica was recently described as a new member of the genus *Francisella*, and the clinical isolate used in this study, LADL 07-285A recovered from moribund tilapia in Costa Rica (57), was found to share more than 99% homology with the *F. asiatica* by sequence comparison of the 16S rRNA genes.

As previously described by several authors, a wide variety of mammalian and fish bacterial pathogens are resistant to normal serum killing, whereas nonvirulent strains of Gram-negative bacteria and capsule and/or lipopolysaccharide (LPS) mutants are generally susceptible to the bactericidal activity of the serum (2, 8, 9, 27, 62). In this study, it was demonstrated that both the *F. asiatica* wild type and a Δ*iglC* mutant are resistant to the action of the complement in tilapia serum. Recent work on the human pathogen *F. tularensis* demonstrated that the bacterium is resistant to serum killing but requires complement factor C3-derived opsonins for uptake by phagocytic cells and subsequent intracellular growth (12). Those data suggest that important virulence factors for *F. tularensis* are its ability to bind the complement regulatory glycoprotein factor H and inactivation of C3b to iC3b, which culminates in opsonin-induced uptake for subsequent intracellular growth. The C3b inactivation also leads to inefficient membrane attack complex assembly, which contributes to the ability of this bacterium to resist complement lysis. While it is clear that *F. asiatica* isolates are able to survive killing by serum, it is still unknown if all *F. asiatica* and *F. noatunensis* isolates share the same mechanism of survival as *F. tularensis*.

The capability of *F. tularensis* to multiply intracellularly is well documented in insects, as well as in a broad range of mammals, including rabbits, rodents, beavers, and humans (19). In the case of *F. asiatica*, the ability to survive inside a wide variety of fish cells was hypothesized, but this conclusion was based only on histopathological analysis of infected tissue in natural cases (57).

Macrophages are generally a population of ubiquitous mononuclear phagocytes that are responsible for numerous homeostatic, immunological, and inflammatory processes (19, 52). The ability to survive intracellularly is crucial for several bacterial fish pathogens after invasion of their eukaryotic target cells (16, 22, 41). Distinct subpopulations of macrophages derived from goldfish (*Carassius auratus*) kidney leukocyte cultures were previously characterized. The subpopulations had distinct morphological, cytochemical, and flow cytometric profiles and also differed in their antimicrobial functions after activation with macrophage activation factors (MAF) and bacterial lipopolysaccharide (LPS) (11, 47, 59). Similar results were found when tilapia HKDM were analyzed by flow cytometry and light microscopy (data not shown). Five-day-old HKDM were bigger and morphologically similar to mature tissue macrophages of mammals, while the 4-h-old HKDM appeared as round cells with eccentrically placed nuclei that resembled more a mammalian monocyte (11, 47, 59).

In the present study, the *F. asiatica* wild-type strain was found to be capable of intracellular survival and replication within both 4h-HKDM and 5d-HKDM from tilapia. Effective internalization by both cell types was partially mediated by a heat-sensitive serum component, presumably complement. Complement and/or complement receptors (CR) have been associated with efficient internalization of many mammalian and fish pathogens, including *F. tularensis*, *Mycobacterium* spp., *Listeria monocytogenes*, and *Edwardsiella ictaluri* (6, 12, 13, 16, 22). With the *F. tularensis* live vaccine strain (LVS), optimal phagocytosis by dendritic cells (DC) is dependent on complement factor C3-derived opsonins and the major complement receptors expressed by DC, the integrins CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (12).

Uptake of *F. asiatica* by both 4h-HKDM and 5d-HKDM

following opsonization with tilapia NS was significantly greater than uptake following pretreatment with HINS (Fig. 4), indicating involvement of complement and the complement receptor. In 4h-HKDM, uptake remained at 40% of NS uptake when HINS was used to pretreat the bacteria (Fig. 4), indicating that uptake was only partially mediated by complement, similar to the situation for *F. tularensis* (3, 57). In 5d-HKDM with HINS pretreatment, however, uptake was significantly lower than for 4h-HKDM with HINS, at only 5% of NS uptake, indicating that either 5d-HKDM increased expression of the CR or there is increased affinity for complement components compared to 4h-HKDM. Further work with *F. tularensis* (6, 54) demonstrated that monocyte-derived macrophages (MDM) phagocytose more *Francisella* than monocytes, with a major contribution from the mannose receptor on MDM. When using NS to opsonize *F. asiatica*, pretreatment of 4h-HKDM with mannan reduced uptake to 55% of NS, indicating a substantial involvement of the MR, with an even larger decline with mannan-treated 5D-HKDM, at 30% of NS. This is similar to the case for *F. tularensis*, where mannan pretreatment had a greater effect on uptake by MDM than on that by monocytes, although the effect of mannan pretreatment of tilapia 4h-HKDM on *F. asiatica* uptake was greater than seen for *F. tularensis*. Uptake of *F. asiatica* by tilapia 4h-HKDM was reduced to 20% of NS when mannan pretreatment was applied and bacteria were pretreated with HINS, indicating that CR and MR are not the only receptors involved in uptake. Other receptors demonstrated to be involved in the uptake of *F. tularensis* by mammalian macrophages include Fc γ receptors, pulmonary collectin surfactant proteins, and type I and II class A scavengers (6, 51, 54). The difference between tilapia HKDM and the human blood-borne monocytes could be a result of the differential maturation of HKDM, but a clear involvement of both the CR and the MR was observed in both 4h-HKDM and 5d-HKDM. The combination of mannan pretreatment and opsonization with HINS reduced uptake of *F. asiatica* in 5d-HKDM by 99.7% of uptake following NS treatment, indicating that the primary receptors involved are the CR and MR. This is in contrast to the case for *F. tularensis*, where additional receptors were suspected for MDM (54) (Fig. 4).

Our results are consistent with the involvement of the MR in phagocytosis of *F. tularensis*, particularly in 5d-HKDM (Fig. 4). The ligands that engage the MR of *F. tularensis* are unknown; the LPS is a proposed candidate, but the only mannose residues present in the *F. tularensis* LPS are in the core region, which is presumably covered up by the O-antigen repeats. The mannose-containing capsule of *F. tularensis* is also a candidate (28, 29). The LPS of *F. asiatica* also contains mannose in the core, also covered up by the O-antigen repeats (32), but a capsule has not yet been described for *F. asiatica*. Analysis of the *F. asiatica* genome revealed high sequence homology to the *F. tularensis capB* and *capC* sequences, indicating that a capsule might be present as well in this bacterium (E. Soto et al. unpublished data).

Differences between the uptake of *F. asiatica* by 4h-HKDM and 5d-HKDM indicate that the presence or absence of receptors, such as the MR, plays a role in the uptake of bacteria by fish phagocytes. In mammals, uptake of *F. tularensis* by MDM was greater than that by monocytes, presumably because the MR are more abundantly or newly present on mature macrophages than on monocytes. More research is needed to elucidate the role of different bacterial receptors in fish mononuclear cells. After uptake, regardless of whether the bacteria were preopsonized with normal serum or heat-inactivated serum, intracellular replication was equal in either HKDM population (data not shown), similar to the situation in *F. tularensis* (12).

Previously, we identified the *iglABCD* operon in the fish isolate *F. asiatica* LADL 07-285A and demonstrated that *iglC* is required for virulence in the fish host (58). In this study, we show that *iglC* is required for intracellular survival and growth in tilapia HKDM. Similar results have been obtained with *F. tularensis*, where Δ*iglC* mutant strains are defective for survival and replication within mammalian macrophages. Expression of *iglC* was induced during growth of *F. tularensis* in macrophages and was required for intracellular multiplication in macrophages and for virulence in mice (25, 33, 38, 53). Inactivation of the *iglC* and *mglA* genes of *F. tularensis* also abolishes its capacity to escape from the phagosome into the cytoplasm and to multiply intracellularly in mouse peritoneal exudate macrophages (38, 53).

After 24 h of infection with *F. tularensis*, the murine macrophage-like cell line J774.A1 underwent apoptosis and pronounced cytopathogenesis. Further work by the same group demonstrated that an *F. tularensis* Δ*iglC* mutant did not induce apoptosis in infected cells, suggesting an involvement of IglC in the induction of apoptosis in *F. tularensis*-infected macrophages (35). Similar results were found for *F. asiatica* in this study, with significantly greater LDH levels in supernatants of tilapia HKDM infected with the wild-type and the IglC-complemented *Francisella* strains than in those infected with the Δ*iglC* mutant. The Δ*iglC* mutant strain also induced significantly lower caspase-3/7 activity, to levels similar to those in the uninfected cells.

Apart from the advantages that microbes gain from controlling host cell apoptosis, it has been suggested that apoptosis functions as a host defense mechanism by depriving microorganisms adapted to the intracellular environment of their preferred habitat (34, 35). As previously described for *F. tularensis*, *F. asiatica*-mediated apoptosis occurred at a later stage of *in vitro* infection in macrophages than that described for *Salmonella*, *Yersinia*, *Shigella*, or *Legionella* (34, 35). As previously suggested, the delayed apoptosis induced by *Francisella* spp. would allow the bacteria to replicate within the target cells, and that subsequent induction of apoptosis allows them to escape when nutrients become limiting (35).

The initial uptake of *F. tularensis* occurs by looping phagocytosis, in which the bacterium is engulfed in a spacious, asymmetric pseudopod loop (18, 19). A similar process was not observed for *F. asiatica*, but only limited cells were observed. After uptake, *F. asiatica* appears to reside within a tight membrane-bound phagocytic vacuole (Fig. 6A and B). As previously described, *F. tularensis* resides within membranes containing discrete, easily identifiable lipid bilayers measuring between 25 and 34 nm immediately after infection (18, 19). Although not fully characterized, a clear, tightly membrane-bound phagocytic vacuole surrounds internalized *F. asiatica* (Fig. 6A and B). Similar to the case for *F. tularensis*, the phagosomal membranes of some vacuoles containing *F. asi-*

atica are disrupted, allowing *F. asiatica* to escape to the cytoplasm, but some bacteria replicate in spacious vacuoles (Fig. 6D to F). At 8 to 12 h postinfection, most *F. tularensis*-containing vacuoles are fragmented and the majority of the bacteria are free in the cytoplasm (4, 18, 19). Although some of the *F. asiatica* organisms were observed free in the cytoplasm at 12 h postinoculation, the majority of bacteria were found in spacious vacuoles (Fig. 6D to F). Further work is needed to completely elucidate the location of *F. asiatica* in HKDM at later time points of infection.

In conclusion, the results indicate that *F. asiatica* is able to resist complement-mediated lysis and to survive and efficiently replicate in 4h-HKDM and 5d-HKDM, whereas a Δ*iglC* mutant was deficient in intramacrophage growth. The mutant remained resistant to complement but failed to release significant amounts of LDH or to induce significant activity of caspases 3 and 7.

The pathology and immune response to acute *Francisella* infection in zebrafish were recently described, and it was demonstrated that there are many features in common with infections in mammals (60), suggesting the zebrafish system as a model for studying *Francisella* infection. Infection in the zebrafish, however, required intraperitoneal injection of 10^6 CFU to cause 100% mortality in 5 days, while 3.45×10^5 CFU resulted in only ~2% mortality (60). In contrast, as few as 23 bacteria injected in the peritoneum are capable of causing mortalities in tilapia, and even fewer are enough to cause serious pathological lesions in important organs such as the head kidney and spleen (58). Macrophage studies are difficult in zebrafish because of their small size, so comparative analysis of intracellular pathogenesis cannot be done. Given the highly virulent infection in tilapia (similar to that of *F. tularensis* in mammals), the similarity of intracellular replication, and the high degree of homology between *F. tularensis* and *F. asiatica* virulence gene sequences, including the *iglABCD* operon and type VI secretion genes (*dotU*, *vgrG*, and *iglAB*), we suggest that *F. asiatica* infection in tilapia could be used as a model for tularemia in mammals.

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